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Bioavibility Studies of Ocular Gels of Pilocarpine Microspheres

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Abstract: Egg albumin microspheres containing pilocarpine nitrate were prepared by heat stabilization method. Factors affecting size and encapsulation efficiency were optimized to obtain microspheres in the size range 1 to 12 µm to make them undetectable by eyes and big enough to entrap drug efficiently. Encapsulation efficiency was found to be 62.5%. *In vitro* release of drug from microspheres followed spherical matrix mechanism. Microspheric gels were prepared by triturating drug-loaded microspheres with Carbopol-940 gels. Biological response of microspheric gels and suspension were measured by reduction in intraocular pressure. Comparative evaluations were made by preparing plain gels of same drug concentration. Bioavailability parameters of all the formulations were studied and comparisons were made.

Keywords: Egg albumin, microspheres, pilocarpine nitrate, intraocular pressure, carbopol gel

1.Introduction

One of the major problems of ophthalmic therapy is to provide and maintain an adequate concentration of the drug at the site of action for prolonged period of time. Conventional eye drops deliver drug immediately after instillation, followed by rapid decline to insignificant levels, usually within minutes. Most topically applied drugs are extremely limited in their ability to penetrate various anatomical and physicochemical barriers. Hence there is a need of effective topical formulation capable of promising drug penetration and maintaining therapeutic levels within reasonable frequency of application.

The addition of suitable polymers to liquid ophthalmic vehicles is a common method for increasing the ocular contact time and hence the drug bioavailability [1-2]. However, the relatively less viscous ophthalmic solutions generally do not have sustaining effect. Gels or slowly dissolving lamellae remain in the eye for extended periods of time, but their high water content allow a drug substance to diffuse quickly out of the system [3-4]. A new approach to achieve controlled drug delivery is to use liposomes [5-6], nanoparticles [7] or microspheres [8], as drug carriers. Such carriers can potentially alter the residence time in the precorneal area, control the rate of release and affect the drug interaction with the corneal epithelium.

Glaucoma is an ocular disease characterized by an increase in intraocular pressure (IOP). Pilocarpine nitrate (PN) was selected for the studies because of its ease of analysis and its effect in lowering intraocular pressure, which reduces the need for preparing theoretical model for *in vivo* analysis.

The use of natural biodegradable polymer to deliver drugs continues to be an area of active research, despite the advent of synthetic biodegradable polymers. Some of the materials taken from nature for microspheric preparation includes lipid and waxes [9-10], protein like albumin and gelatin [11 -12], polysaccharides like alginate and chitosan [13-14]. In the present study Egg albumin was chosen to prepare microspheres in the size range 1-12 µm to remain undetectable by the eyes and big enough to entrap drug efficiently.

The present study had the purpose of evaluating the PN loaded albumin microspheres and Carbopol (C-940) gels aimed at ensuring a prolonged medication in the eye. Gels were prepared to provide a protective coating on drug loaded albumin microspheres and hence reduce the diffusion of drug from microspheres. Patient compliance was also improved due to formation of the gel.

2. Materials and Methods

Pilocarpine nitrate was a gift sample from JT Baker Chemicals Co., Philipsburg NJ, and was analyzed in the laboratory. Egg albumin was received from Loba Chemie, Mumbai, India, and olive oil was purchased from Ashwin Chemicals, Mumbai, India, and used as obtained. Ether and liquid paraffin were procured from SD Fine Chemicals Ltd., Mumbai, India, and all other chemicals and reagents used were of analytical grade. All *In vivo* studies were performed in the laboratories approved by animal ethical committee at C.U.Shah college of Pharmacy, Santacruz (W),Mumbai.

2.1.Method of preparation

Albumin microspheres were prepared by protein gelation process [15]. Egg albumin was dissolved in distilled water. This solution was added drop wise in olive oil to make an emulsion. The emulsion so formed was added drop wise in the preheated olive oil (125 $\pm 5^{o}$), stirred at 1500 g. After heat stabilization time of 10 min, the preparation was cooled to 25^{o}, centrifuged at 2500 g, and supernatant was decanted. Microspheres thus obtained were washed with liquid paraffin and twice with ether to get a free flowing and discrete product. The same were then suspended in anhydrous ether and stored at 4^{o} in an airtight container. Numbers of variables were studied affecting size, shape and entrapment efficiency of microspheres. Separate batches were prepared and minimum of 100 particles were observed under optical microscope using oil immersion lens to optimize the variables.

2.2.Optimization of process variables

Variables such as concentration of albumin, stirring rate during emulsification [16-17], viscosity of oils and drug concentration were studied by preparing series of batches.

Other factors such as emulsion drop rate, heat stabilization temperature, stirring rate during heat stabilization of microspheres and heat stabilization time were also studied and observations were recorded. Three batches of microspheres in olive oil at 105^{o}C, 125 ^{o} and 145 ^{o} were prepared keeping other variables same as described and their release rates were studied (fig.2).

2.3.Analysis of surface drug

To a portion of ether suspension of microspheres equivalents to 5 mg of PN, Tween 80 (0.05 ml) was added and the suspension was gently vortexed. Ether was then evaporated and 10 ml of 0.5 N HCl was added, centrifuged at 3000 g for 5 min and supernatant was analyzed spectrophotometrically at 215 nm.

2.4.Analysis of entrapped drug

Microspheres obtained after washing were digested in 10 ml of 0.5 N HCl overnight. This solution was centrifuged to get a clear supernatant that was suitably diluted with 0.5 N HCl and assayed for PN content spectrophotometrically at 215 nm.

2.5.Determination of *in vitro* release of PN from microspheres

Drug release was determined with the help of modified USP XXI dissolution rate model[18]. A 250 ml beaker was placed in the vessel. Plastic tube of diameter 17.5 mm opened from both the ends was tied at one end with treated cellophane membrane and dipped into the beaker containing dissolution media. Paddle type stirrer was attached in the center of the beaker and the speed was maintained at 100 g. The beaker was filled with 90 ml phosphate buffer (pH 7.4) and temperature was maintained at 37±1°. Drug loaded microspheres were suspended in 10 ml of phosphate buffer.

Samples were withdrawn periodically for 8 h and concentration was determined spectrophotometrically at 215 nm. Similar studies were performed using plain drug and the data was plotted [19].

2.6.Preparation of gel

Weighed quantity of Carbopol (C-940) was dispersed in water. This dispersion was neutralized with sodium hydroxide solution (18% w/v) to give a transparent gel. Viscosity was measured with Brookfield Synchrolectric Viscometer Model RVT with Spindle D at 5 rpm (pH 7.6) and at 25^{0}.Gels in the concentration of Carbopol 1%, 2% and 4% w/w with and without drug were prepared [20]. Phenyl mercuric nitrate in the concentration of 0.004% w/w was added as a preservative. Gels were sterilized by autoclaving at 121^{0} for 30 min and studied the viscosity. Biological responses were observed in albino rabbit eyes [21] and observations were recorded.

2.7.Preparation of microspheric gel:

Weighed quantity of albumin microspheres were gently mixed with previously sterilized Carbopol gel (blank) to prepare microspheric gels containing an equivalent quantity of 1% PN w/w. Drug containing microspheres were found to be swelled and lost their structure when sterilized by autoclaving, hence sterilized gels were used. Three formulations were prepared containing 1%, 2% and 4% Carbopol w/w with 1% PN microspheres w/w, and reduction in IOP was measured in albino rabbit eyes.

2.8. *In vivo* animal studies

In vivo studies were conducted in albino rabbits (Haffkine strain) of either sex weighing between 1.8

and 2.5 kg. All experiments were carried out at room temperature. Minimum of four rabbits were used in each experiment. Reduction in intraocular pressure (IOP) was measured by Shioetz tonometer [21]. Minimum two readings of IOP were taken prior to administration of gels, which was denoted as I_0 .The formulation (0.05 ml) was administered with the help of an insulin syringe in the lower cul-de-sac of one eye. The control (0.05 ml) was administered in the other eye. Reduction in IOP at time t was denoted as I_t and observations were recorded. Graph was plotted as I_{rt} v/s t where $I_{rt} = I_t - I_0 / I_t$. Same animals were used repeatedly allowing minimum two days between two successive experiments.

3.Results and Discussion

Albumin microspheres containing pilocarpine nitrate were prepared by simple emulsion technique. Various studies have been performed to obtain desirable variables. Albumin concentration was studied due to the fact that albumin plays a vital role as emulsifying agent, thus affects the droplet size and stability [22]. Higher albumin concentrations produced smaller microspheres. However, it was not possible to increase the concentration of albumin beyond 400mg/ml (Table.1).

It has been reported that the power of emulsification and emulsification time affect the droplet size of emulsion [23]. Longer duration of emulsification and greater emulsification power though resulted in smaller microspheres, stirring speed higher than 1600rpm and emulsification time more than 10min resulted in frothing.

Fixed oils with different viscosities viz olive, coconut and groundnut oil were used to obtain optimum size of microspheres. Olive oil with viscosity 120.6 cp produced smallest size of microspheres (average particle size 4.56) whereas; groundnut oil (viscosity 169.0 cp) and coconut oil (viscosity 130.1 cp) had produced microspheres of average particle size 5.57 and 5.37 µ respectively. Four batches of microspheres were prepared having 5%, 10%, 12.5% and 15% w/w drug concentration, calculated on the basis of albumin used. Uniform difference in the microsphere size was seen in all the batches. However, the batch containing 12.5% w/w drug showed maximum entrapment efficiency with desirable mean particle size of 4.58 µ. Effect of heat stabilization temperature on microsphere size was studied by conducting experiments at various temperatures such as 105, 125 and 145°. A heat stabilization temperature of 105° produced larger particle size with some water trapped in the albumin matrix. At temperature 125° microspheres obtained were of desirable size. Microspheres prepared at 145° were harder with very slow drug release rate. Other variables studied were heat stabilization time and emulsion drop rate. Heat stabilization for more than 10 min and drop rate lesser than 80 ± 10 per min resulted in charring of microspheres. Observations are summarized in (Table.1)

Photomicrographs were taken on the transmission electron microscope (model 100S, Geol Ltd., Tokyo, Japan), for morphological studies of microspheres [15]. Electron microscopic studies were carried out by placing a drop of microsphere sample on a copper grid that was dried in an oven at 60°. Copper grid was coated with phosphotungustic acid (1% w/v) solution and the samples were observed under TEM at 8,000 X. (fig.1).

Release rate studies of PN microspheres were performed on specially designed *in vitro* release rate model for 8 h. Drug was analyzed spectrophotometrically in triplicate samples. (fig. 2) represents the plots of pilocarpine released from albumin microspheres according to spherical matrix mechanism[19]. Function $3/2 [1 - (1-Q)^{2/3}] - Q = F(t)$ showed the straight line, hence the release of PN from albumin beads complies with the dissolution model of spherical matrix [18].

Comparative studies were conducted using PN solution, PN microspheric suspension, plain PN gels and microspheric gels. Table.2 reveals various pharmacokinetic parameters of 1, 2 and 4% PN w/v marketed solution (Pilocar®) manufactured by FDC (India Ltd).. Increase in the concentration of PN from 1% to 2% w/v causes a significant increase in biological response. Further increase in concentration from 2% to 4% w/v increases the magnitude of the response but not the duration. Time required to achieve peak biological response is same in all the three formulations, where as $I_{rt(max)}$ increased from 0.431 to 0.565 with increase in concentration of PN from 1% to 4% w/v. AUC was also increased from 1% w/v (6.5) to 4%w/v (12.45) (Table .2).

Carbopol gels (1, 2 and 4% w/w gels were prepared containing 1% w/w PN. Gels were evaluated for their rheological properties. Viscosity and adhesivity increased with increase in concentration of Carbopol (Table. 3). Addition of PN causes decrease in viscosity of C-940 gels. The same effect can be confirmed from the biological studies[24] .

Table.4 reveals the studies of gels containing 1%, 2% and 4% w/w carbopol all containing 1% w/w PN. Carbopol gel (1% w/w) showed DR of 240 min as compared to DR of 280 min with 2% w/w carbopol gel. An increase in carbopol concentration from 1% w/w to 4% w/w caused increase in DR from 240 min to 300 min. AUC was also observed to increase from 12.09, 14.21 to 17.35 with the increase in the concentration of Carbopol from 1%, 2% to 4%w/w. Similar results were obtained by Schoenwald *et al.*[20], in their studies of different gel formulations containing

pilocarpine nitrate. The increases in viscosity of solutions causes decrease in drainage and hence increase in bioavailability. But this increase in bioavailability is not to same extent as increase in viscosity caused only two-fold increase in the aqueous humor drug concentration [24-25]. This was attributed to shear thinning of pseudoplastic gels due to blinking and movement of the eye [26].

Schoenwald R. D. and co-workers[20] shown in their studies that extended duration of miotic response obtained with C-940 and ethylene maleic anhydride gel containing pilocarpine hydrochloride was better explained on the basis of high yield values obtained with these gels. For plastic fluid, yield values indicate minimum-shearing force required before flow starts. Thus gels, with high yield value may not flow with slight shearing in the eye and hence may be better retained in the eyes.

Three formulations *viz* 1%, 2% and 4% w/w carbopol gel containing 1% PN microspheres were prepared and various pharmacokinetic parameters were noted (Table.5). Formulation containing 4% w/w Carbopol was excluded due to its high viscosity and inconvenience in its application. The AUC obtained by 2% microspheric carbopol gel was more (AUC 36.78) as compared to 1% (AUC 31.54). Duration of response was also maximum with 2% PN M gel (750 min) in comparison with 1% PN M gel (525 min).

All C-940 gels containing PN showed higher DR values as compared to conventional eye drop containing the same amount of PN (1% w/w). Table.6 shows the comparative studies of bioavailability parameter of PN solution, suspension, gel and microspheric gel. Suspension of PN microspheres prolonged the biological effect in comparison with aqueous solution of the free drug. A delayed time of peak (TP 120 min) with longer duration of action (DR 420 min) and an increase in AUC (20.743) was obtained when colloidal carriers were used. Similar results were obtained in the studies of the kinetics of *in vitro* release and the pharmacokinetics of gelatin and albumin microspheres with pilocarpine in rabbit by Sorin *et al.*[19]. Bioadhesion may also play role in improving ophthalmic bioavailability by improving corneal contact time since carbopol resins are known to possess bioadhesion.

4.Conclusion

The increase in AUC suggests that the bioavailability of PN can be further improved by incorporation of drug into microspheres and subsequently in gels.

TABLE 1: OPTIMUM VARIABLES IN THE PREPARATION OF ALBUMIN MICROSPHERES

Variables	Ideal condition
Albumin concentration	Aqueous solution of egg albumin 400mg/ml
Drug concentration	Aqueous solution containing 50mg/ml of pilocarpine nitrate
Rate of stirring during emulsification	1200 rpm
Emulsification time	5.0 min
Stirring rate during heat stabilization	1600 rpm
Heat stabilization temperature	125^{o}
Oil	Olive oil
Emulsion drop rate	80 ± 5 drops per min
Heat stabilization time	10 min

Albumin microspheres in the size range 1-12 µm were obtained by selection of several variables as shown above.

TABLE 2: MAIN BIOAVAILABILITY PARAMETERS OF SOLUTIONS

Formulations	TP	I_{rt} (max)	DR	AUC
PN solution 1%	30	0.431	155	6.5
PN solution 2%	30	05025	240	11.6
PN solution 4%	30	0.565	225	12.45

TP shows the time required to achieve maximum reduction in IOP, DR ,the duration of significant reduction in IOP (I_{rt} = 0.1) and AUC is area under the curve.

TABLE 3: VISCOSITY AND YIELD VALUE OF CARBOPOL-940 GELS

Formulation	PN conc %	Viscosity Cp x 10^4		Yield value
		without PN	With PN	
Plain gel (Carbopol)				
1%	1	6.2	5.58	----
2%	1	8.8	8.0	----
4%	1	13.12	12.95	4280
PNM carbopol gel				
2%	1	---	11.5	3480
4%	1	---	14.5	4440

Brookfield Synchrolectric viscometer model RTV was used with spindle-T-D at speed 5 rpm (25^0)

TABLE 4: MAIN BIOAVAILABILITY PARAMETERS OF CARBOPOL GELS CONTAINING 1% PN

Formulation	TP(min)	I_{rt}(max)	DR (min)	AUC
Carbopol Gel 1% w/w	30	0.346	240	12.039
Carbopol Gel 2% w/w	30	0.468	280	14.271
Carbopol\Gel 4% w/w	30	0.473	330	17.351

TP shows the time required to achieve peak reduction in IOP, I_{rt} (max), the time required to achieve maximum reduction in IOP (I_{rt} = 0.1) DR is duration of response and AUC is area under the curve.

TABLE 5: MAIN BIOAVAILABILITY PARAMETERS OF MICROSPHERIC GELS CONTAINING 1% PN (W/W)

Formulations	TM (Min)	TP (Min)	$I_{rt(max)}$	DR (Min)	AUC
PNM Gel 1% Carbopol (w/w)	10	240	0.472	525	31.545
PNM Gel 2% Carbopol (w/w)	10	120	0.522	750	36.787
PNM Gel 4% Carbopol (w/w)	10	120	0.511	720	30.6

TM is time required to achieve significant miotic response (I_{rt} = 0.1), TP shows the time required to achieve significant reduction in IOP, I_{rt} (max) is maximum response, DR is duration of response and AUC is area under the curve.

TABLE 6: COMPARATIVE EVALUATION OF BIOAVAILABILITY PARAMETERS OF VARIOUS FORMULATIONS CONTAINING 1% PN

Formulations	TP (Min)	$I_{rt(max)}$	DR (Min)	AUC
PN Solution	30	0.431	155	6.5
2% plain Carbopol Gel	30	0.468	240	14.21
PN suspension	120	0.502	420	20.16
2% Microspheric Gel	120	0.522	750	36.78

TP shows the time required to achieve significant reduction in IOP (I_{rt} =0.1), TP shows the time required to achieve peak reduction in IOP and I_{rt} (max) is maximum response.

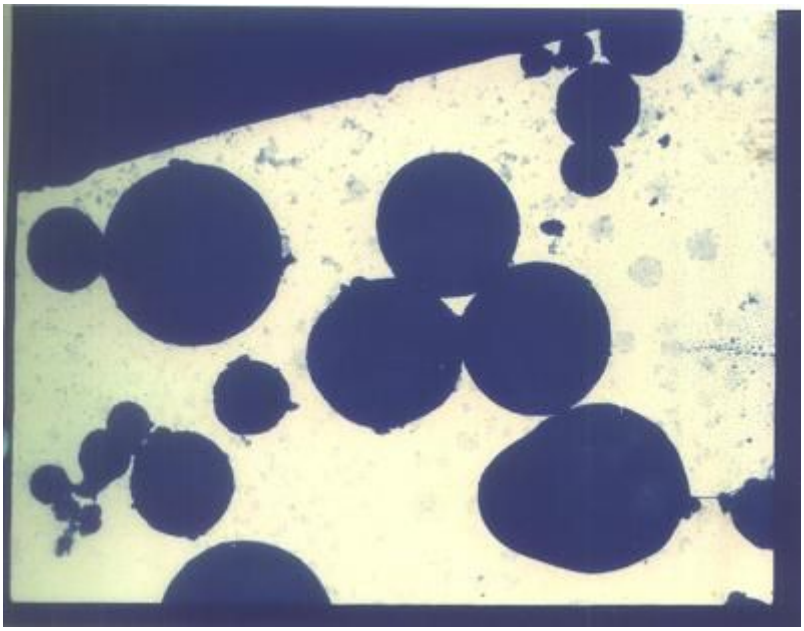

Fig. 1: TEM photograph of albumin microspheres.
Microspheres were stained by 1% w/v solution of Phosphotungustic acid

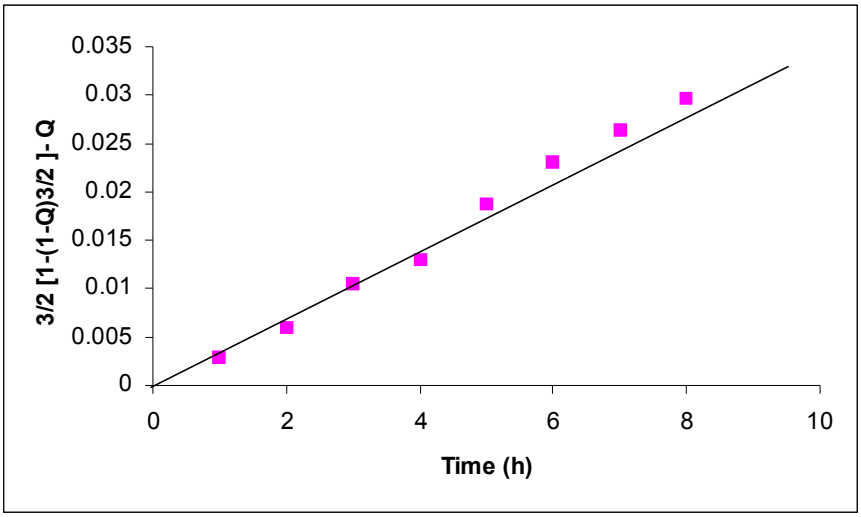

Fig. 2: Plot of 3/2 $[1-(1-Q)^{2/3}]$ – Q versus time.
Straight line shows the dissolution of pilocarpine from microspheres follows spherical matrix mechanism

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References
1. Saettone, M.F., Giannaccini, B., Ravecca, S., Marca, F.L., and Tota, G., Polymer effects on ocular bioavailability- Influence of different liquid vehicles on the mydriatic response of tropicamlde in human and in rabbits, Int J Pharm, 1984, 20, 187-202.
2. Saettone, M.F., Giannaccini, B., Teneggi, A., Savigni, P., and Tellini, N., Vehicle effect on ophthalmic bioavailability: the influence of different polymer on the activity of pilocarpine in rabbit and man, J Pharm Pharmacol, 1982, 34, 464-466.
3. Thomas, F., Patton Joseph, R.,and Robinson., Ocular evaluation of polyvinyl alcohol vehicle in rabbits, J Pharm Sci, 1975, 64(8**),** 1312 – 1316.
4. Bensinger, R., Shin, D.H., Kass, M.A., Podos, S.M.,and Becker, B., Pilocarpine ocular inserts. Invest Ophthal Vis Sci, 1976, 15, 1008-1010.
5. Singh, K.,and Mezei, M., Liposomal ophthalmic drug delivery system I. Triamcinolone acetonide, Int J Pharm**,** 1983, 16(3), 339-344.
6. Robert, E.S., Jr. Denise.,Yang, C., Mark, A.R., and Vincent, HLL., Effects of topically applied liposome's on disposition of epinephrine and inulin in the albino rabbit eye, Int J Pharm 1983,13(3), 263-272.
7. Widder, K.J., Flouret, G.,and Senyie, A.E., Albumin Nanoparticles, J Pharm Sci, 1979, 68, 79
8. Siman, B., Natural Polymers as drug delivery system, In: Mark C., Robert L., editors, Biodegradable polymers as drug delivery systems. Marcel Dekker, New York, 1990,231-259.
9. Adeyeye, C.M., and Price, J.C., Development of evaluation of sustained- release ibuprofen-wax microspheres I effect of formulation variable on physical characteristics. Pharm. Res, 1991, 8,1377-1383.
10. Karasulu, E.H., Karasulu, Y., Ertan, G., Kirilmaz, L., and Güner, T., Extended release lipophilic indomethacin microspheres: formulation factors and mathematical equations fitted drug release rates, Eur J Pharm ScI, 2003, 19 (2-3), 99-104.
11. Giunchedi, P., Maggi, L., Torre, M.L., and Conte U., Spray dried albumin microspheres containing carbamazepine Proceed, Int Symp Control Rel Bioact Mater, 1994, 21, 622-623.
12. Saparia, B., Murthy, R., and Solanki, A., Preparation and evaluation of chloroquine phosphate microspheres using cross-linked gelatin for long term Drug delivery, Ind J Pharm Sci, 2002, 64(1), 48-52.
13. Bregni, C., Degrossi, J., Garcia, R., Lamas, M.C., Firenstein, R.D., and Aquino M. A., Microspheres of bacillus subtilis, Ars Pharm, 2000,41, 245-248.
14. Wang, Y.M., Sato, H., Adachi, I., and Horikoshi, I., Optimization of the formulation design to chitosan microspheres containing cisplastin, J Pharm Sci, 1996, 85(11), 1204-1210.
15. Gupta, P.K., Hung, C.T.,and Perrier, D.G., Albumin microspheres I release characteristics of adriamycin, Int J Pharm**,** 1986, 33(1-3), 137-146.
16. Lee, K.C., Koli, I.B., Kim, W.B., and Lee, Y.J., Size and morphological analysis of albumin microspheres in the lungs and liver of mice, Int J Pharm,1988, 44, 49.
17. Wand, Y.M., Sato, H., and Adachi, H.,Optimization of the formulation design of chitosan microspheres containing cisplastin, J Pharm Sci, 1996, 85(11), 1204 – 1210.
18. Jun, H.W., Lai, J.W., Preparation and *in vitro* dissolution test of egg albumin in microcapsules of nitrofurantoin, Int J Pharm, 1983, 16, 65.
19. Sorin, E.L., The kinetics of *in vitro* release and the pharmacokinetics of mitotic response in rabbits of gelatin and albumin microspheres with pilocarpine, Int J Pharm, 1989, 54, 71-78.
20. Schoenwald, R.D., Ward, R.L., De Santis, L.M., Roehrs, R.E., Influence of high viscosity vehicles on miotic effect of Pilocarpine, J Pharm Sci, 1978, 67, 1280.
21. Zimmer, A.K., Zerbe, H.,and Kreuter, J., Evaluation of pilocarpine loaded albumin particles as drug delivery systems for controlled delivery in the eye I. *In vitro* and *in vivo* characteristics, J Cont Rel, 1994, 32, 57–70.
22. Higuchi, W.I., Okada, R.,and Lemberger, A.P., Aggregation in oil-in-water emulsions. Effects of dioctyl sodium sulfosuccinate concentration, J Pharm Sci, 51, 683-7.
23. Eberth, K., Merry, J., A comparative study of emulsions prepared by ultrasound and by a

conventional method. Droplet size measurements by means of a Coulter Counter and microscopy, Int J Pharma, 1983, 14 (2-3), 349-353.
24. Hui, H.W.,and Robinson, J.R., Ocular delivery of progesterone using a bioadhesive polymer, Int J Pharm, 1985, 26, 203.
25. March, W.F., Sterwat, R.M., Mandell, A.I., and Burce, L.A., Duration of effect of pilocarpine gel, Arch.Ophalmol, 1982, 100, 1270-1271.
26. Chari, S.S., and Robinson, J.R., Ocular evaluation of methylcellulose vehicle in albino rabbits, J Pharm Sci, 1974, 63, 1218.
27. Deshpande, S.G., Shirolkar, S., Sustained release ophthalmic formulations of pilocarpine, J Pharm Pharmacol, 1989, 41(3), 197-200.

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